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14. ABSTRACT Ovarian Cancer is the leading cause of death from gynecologic cancers in the developed world. We have previously identified a homeobox gene, Six1, which is overexpressed in ovarian cancers as compared to normal ovarian surface epithelium. Overexpression of Six1 is associated with resistance to Tumor Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL) based therapies. We have discovered that this resistance in ovarian cancers is likely related to over-expression of the TRAIL decoy receptor DcR2. On-going studies are evaluating the mechanism and significance of this finding on the way to designing new treatments for ovarian cancer.

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None listed.

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INTRODUCTION:

Ovarian cancer is the leading cause of death from gynecologic cancers in the developed world. Most ovarian cancers are diagnosed late and current treatment results only in a 20% 5-year survival in advanced disease. More effective therapies are urgently needed. One of the most promising therapies in development for ovarian cancer is the use of either the Tumor Necrosis Factor-related Apoptosis Inducing Ligand (TRAIL) or agonistic antibodies that activate the receptors for TRAIL. Both these strategies are designed to induce apoptosis in ovarian cancer cells. TRAIL therapies are particularly exciting because TRAIL reverses chemoresistance to standard chemotherapy as well as having a direct growth inhibitory effect on ovarian cancer cells, while sparing normal ovarian cells. However, the characteristics of ovarian tumor cells that determine whether TRAIL pathway agonists will be effective are poorly understood. For this reason, we currently do not have a rational basis for selecting patients who will benefit most from drugs that target this pathway or for improving the clinical response in those patients whose tumors are refractory to TRAIL pathway activators.

We have previously identified a homeobox gene, Six1, which is over-expressed in ovarian cancers as compared to normal ovarian surface epithelium. Expression of Six1 is correlated with poor clinical prognosis and confers resistance to TRAIL, possibly via upregulation of a decoy receptor. Our original hypothesis was that **"Six1 expression in ovarian cell lines and primary tumor cells results in resistance to TRAIL-induced apoptosis through activation of the DcR1 decoy receptor".** In the first year of the award, DcR1 expression in relation to various Six1 over-expression systems was evaluated and was not found to correlate with Six1 over-expression. However, a related TRAIL decoy receptor, DcR2 was found to increase in Six1 over-expressing cells, and in the second year of the award, further study of this mechanism was planned.

Hence, the specific aims are as follows: (1) to confirm *DcR2* as a downstream target of Six1 in ovarian cancer cells, (2) To determine if *DcR2* expression is the mechanism by which Six1 regulates the response of ovarian cancer cells to TRAIL pathway agonists, and (3) To determine if Six1 expression regulates the response of cell lines derived from primary ovarian cancers to TRAIL pathway agonists. These specific aims are identical to those in the original proposal with the exception of the substitution of DcR2 for DcR1.

In the second year of the award, we planned to verify DcR2 as a downstream target of Six1. We expected that DcR2 (RNA and protein) will be increased with Six1 over-expression (both endogenous and exogenous) and that DcR2 will be decreased with Six1 siRNA knockdown, and that downregulation of DcR2 in Six1 over-expressing cells will restore TRAIL sensitivity.

If our hypothesis is correct, it will have a profound implication for current Phase I studies of TRAIL and its agonistic antibodies in cancers (ovarian and others). Thus, Six1 over-expressing tumors are predicted to be resistant to TRAIL. With this knowledge, it may be possible to predict which cancers are TRAIL insensitive by virtue of their levels of Six1 expression, providing a way to select patients for TRAIL clinical trials that are more

likely to benefit from this therapy. Furthermore, many currently used chemotherapeutic agents exert their cytotoxic effect through activating the TRAIL pathway and TRAIL therapy is synergistic with many chemotherapies. Hence, TRAIL resistance may be a marker for chemotherapy resistance and over-coming TRAIL resistance may render cells sensitive to chemotherapy. Since development of chemoresistance is a major obstacle to successful ovarian cancer therapy, a natural extension of our findings in a subsequent proposal would be to study the effects of reversing TRAIL resistance on the effectiveness of chemotherapy.

BODY:

The following section is organized according to the proposed statement of work for the first and second years of the award and accomplishments towards completing the task.

Task 1. Verify DcR1 as a target of Six1 (1-9 months)

As noted per the year one report, this task was modified to study DcR2 due to the lack of correlation between DcR1 and Six1 and initial data showing a positive correlation between DcR2 and Six1. Hence the specific tasks became:

a. Collect and propagate specimens and cell lines to complete Six1 RNA and *DcR2* RNA and protein analysis.
b. Perform CaOV3-Six1 and SKOV3 SiRNA experiments.

Task 1. Work Completed (in addition to that completed in year 1)

As suggested above, a panel of ovarian cancer cell lines with various endogenous Six1 levels [1-7] were propagated and analyzed for Six1 expression and DcR2 expression by quantitative real time PCR (qRT-PCR). TRAIL sensitivity was also determined for each cell line (sensitive = $IC_{50} \leq 50$ ng/ml, resistant = $IC_{50} > 50$ ng/ml). Results are reported in Table 1. Levels of the functional TRAIL receptors DR4 and DR5 were also evaluated as an internal control, since levels of these receptors are not expected to be associated with Six1 expression. Levels of DcR1 did not correlate with Six1 expression as expected and reported in the year one report and are not shown here. DcR2 was 58 ± 12 fg/ng 18s rRNA in cell lines with absent or below mean Six1 expression as opposed to 127 ± 36 fg/ng 18s rRNA in cell lines with above mean Six1 expression ($p= 0.045$ t-test, mean Six1 level =108 fg/ng 18s rRNA). Cell lines were also tested for TRAIL sensitivity by the MTT assay with sensitivity determined as $IC_{50} < 50$ ng/ml TRAIL . As expected, as Six1 levels were increased, cells were more likely to be resistant to TRAIL (absent/below mean level Six1 versus above mean level Six1 as compared to TRAIL sensitive or not sensitive $p= 0.03$ Fisher's Exact test).

Table 1. Endogenous Six1 expression correlates with TRAIL sensitivity and DcR2 expression. A. 15 well characterized cell lines were analyzed for Six1 expression by qRT-PCR (reported as absent/below mean versus above mean relative expression, mean expression = 108 fg/ng 18s rRNA), DcR2 expression (as fg/ng 18s rRNA and TRAIL sensitivity (sensitive = $IC_{50} \leq 50$ ng/ml). B. DR4, DR5 and DcR2 expression was measured in the 15 cell lines and compared to absent/below mean six1 expression versus above mean Six1 expression. Significantly greater DcR2 expression was associated with Six1 expression (p= 0.045 t-test) while functional TRAIL receptor DR4 and DR5 levels were not significantly different.

A.

Cell Line	Description / Reference	DcR2 level (ag/ng 18s rRNA)	Six1 mRNA (absent/below or above mean)	TRAIL sensitive
OVCA 433	Serous Ov Ca / Bast, 1981	126	absent	Yes
OVCA 432	Serous Ov Ca / Bast, 1981	33	absent	No
OVCA 420	Serous Ov Ca / Bast, 1981	26	absent	No
OVCAR5	Ov Ca Ascites / Hamilton 1984	49	below	Yes
2008	Ov Ca / DeSaia, Orth 1994	96	below	Yes
DOV-13	Ov Ca / Duke University 1994	109	below	Yes
Snu251	Endometrioid Ov Ca / Yuan, 1997	13	below	Yes
OVCAR2	Ov Ca Ascites / Hamilton 1984	74	below	Yes
OV1847	Ov Ca / Hamilton, 1990	42	below	No
CaOV3	Ov Ca / ATCC, J. Fogh	21	below	Yes
PECOC167	Serous Ov Ca / Univ of CO , 2008	91	above	No
HeyC2	Serous Ov Ca, passed in mice	254	above	No
Hey	Serous Ov Ca/ Buick, 1985	161	above	No
SKOV3	Grade 2 Ov Ca / J. Fogh, 1977	72	above	No
A2780	Ov Ca / Hamilton, 1990	57	above	No

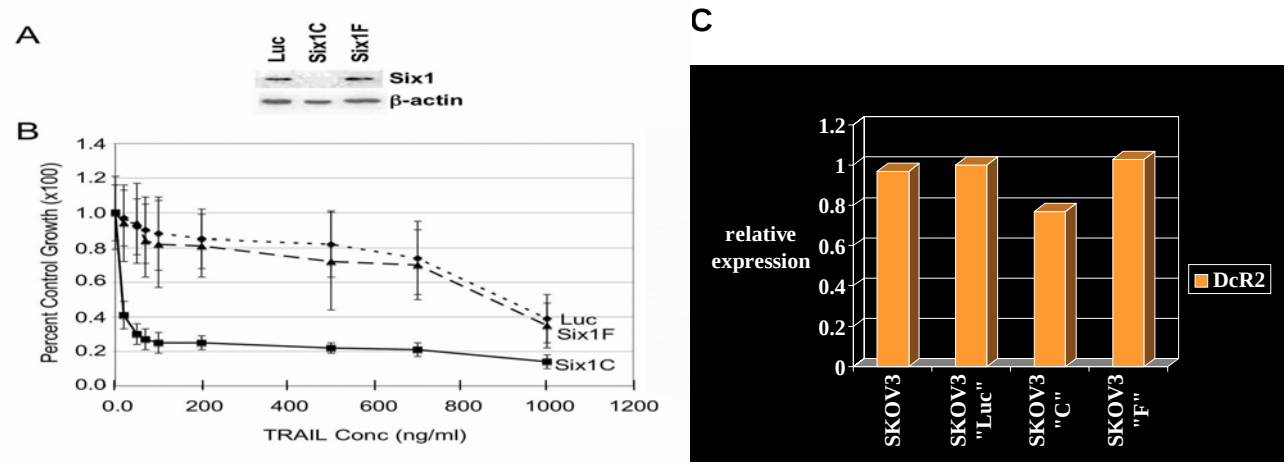
B.

Cell line Six1 Status	DR4 mRNA (ag/ng 18s rRNA)	DR5 mRNA (ag/ng 18s rRNA)	DcR2 mRNA (ag/ng 18s rRNA)
Absent or Below Mean	136±26	186±34	58±12
Above Mean	98±30	257±43	127±36
Significance	NS	NS	P=0.045

To perform a functional protein assay, cell surface expression of the DcR2 receptor was analyzed by flow cytometry using Phycoerythrin (PE) conjugated anti-DcR2 antibody (R&D Systems, Minneapolis, MN). This has been completed and reproducibly repeated so far in 11/ 15 of the cell lines listed in Table 1A. Cell lines with absent or below mean Six1 levels (n=8) demonstrated 5 ± 2 % PE positive cells by flow cytometry as opposed to 43 ± 25% PE positive cells if Six1 was above the mean (p=0.003 t-test). Hence DcR2 is increased in Six1 overexpressing cells, both at the mRNA level and the functional cell surface expressed protein level.

With regard to studying over-expression in isogenic cell lines, CaOv3-Six1 over-expression studies showing associated DcR2 over-expression were reported in year one and as justification for continuing studies or DcR2 as opposed to DcR1. SKOV3-siRNA knockdown studies were performed using SKOV3 siRNA active and sham constructs as reported in our published manuscript [8]. A Figure showing the effects of the control luciferase construct "luc", the sham siRNA construct "F" and the active siRNA construct "C" on TRAIL dose-response from our manuscript is included below as Figures 1A-B. Figure 1A is a western blot for Six1 expression showing baseline high Six1 expression in the "luc" and sham "F" constructs and efficient Six1 knockdown in the "C" construct. Figure 1B shows the associated TRAIL sensitivity resulting from Six1 knockdown. Analysis of DcR2 by qRT-PCR from the parental SKOV3 cell line as well as from the constructs in Figure 1A is shown in Figure 1C. SKOV3 siRNA does cause decreased DcR2, but the magnitude of the decrease in DcR2 is less than what would be expected given the profound effect on TRAIL sensitivity in Figure 1A. This experiment needs to be repeated to generate error bars and to assign statistical significance to the findings.

Figure 1. Effects of Six1 knockdown by siRNA in the SKOV3 cell line on TRAIL sensitivity and DcR2 expression. A. Western blot of Six1 with actin loading control shows efficient Six1 knockdown in the Six1 "C" construct but not the "luc" or Six1 "F" construct. B. Six1 knockdown sensitizes SKOV3 cells to TRAIL as evidenced by leftward shift of the dose-response in the Six1 "C" construct. C. DcR2 expression is decreased (relative expression 0.77) in the Six1 "C" construct as opposed to the parental SKOV3 cell line or the "luc" or Six1 "F" construct cell lines.



Task 2. **Determine whether *DcR2* is a direct or indirect target of Six1.**

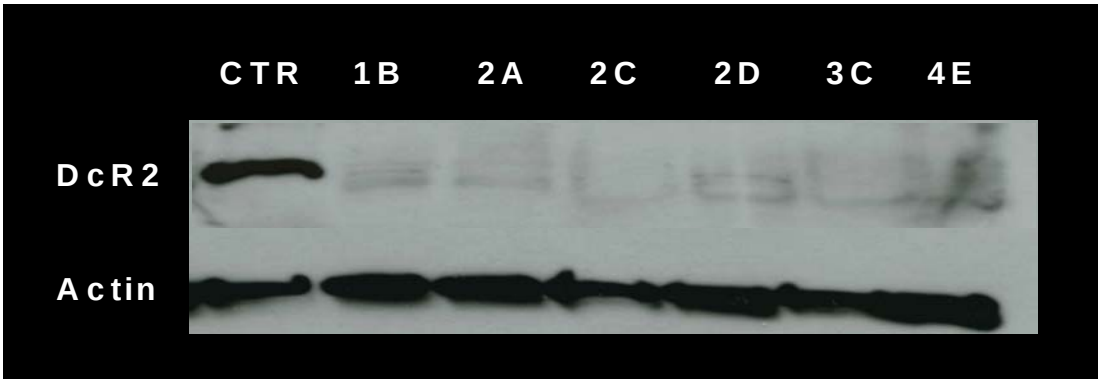
a. Gel shift
b. Chromatin IP experiments
c. Promoter activation studies

To analyze the likelihood of DcR2 promoter binding by Six1, a 2000 bp sequence of the DcR2 mRNA upstream of the DcR2 translation start site was examined for the presence of the "tcagg" consensus Six1 binding sequence[9] and 4 such sequences were found. Oligonucletodies of these regions have been prepared for above experiments.

We also initiated DcR2 knockdown experiments to analyze if the same phenotype seen with Six1 knockdown could be recreated with DcR2 knockdown. This would give functional relevance to any Six1/DcR2 interaction we would find.

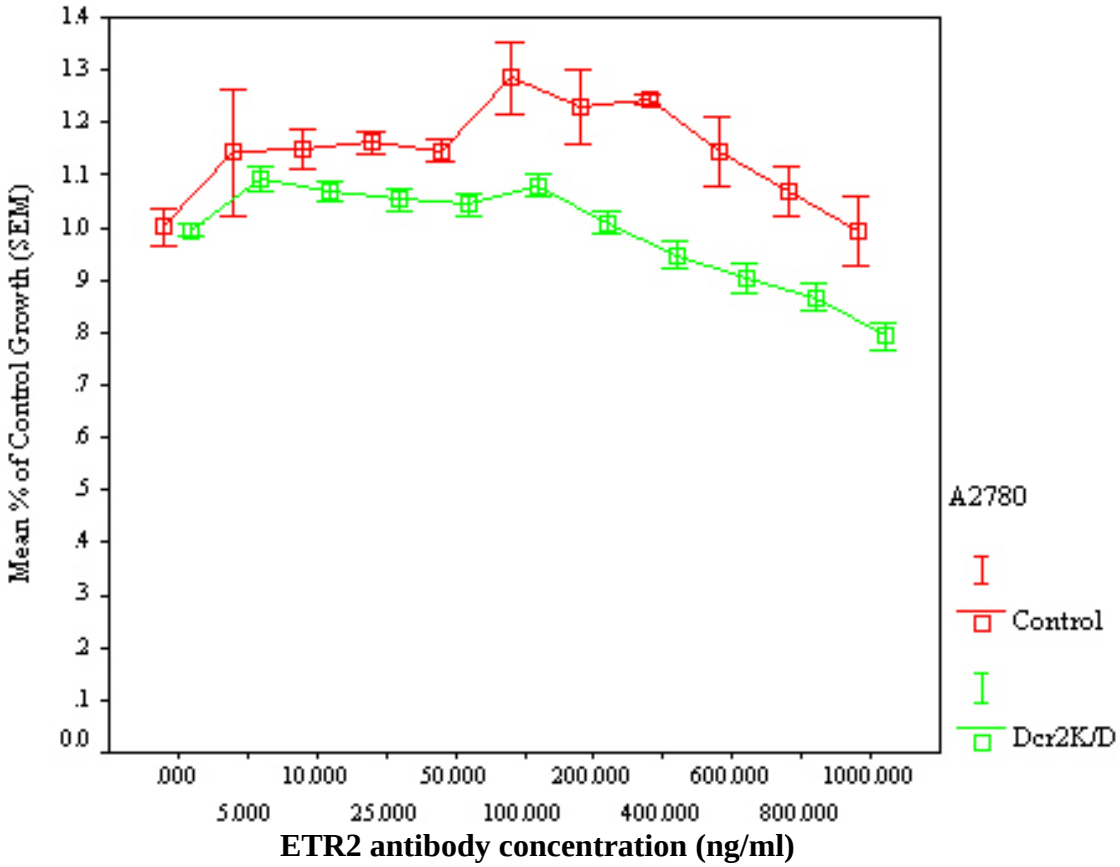
The A2780 cell line over-expresses Six1, has abundant DcR2, and is TRAIL and TRAIL agonistic antibody resistant. The SureSilencing (SABiosciences, Frederick, MD) shRNA knockdown system was used to generate multiple A2780 DcR2 knockdown cell lines using 4 different primer sets (numbered 1-4) and controls. A western blot of DcR2 with control CTR, 3 clones from primer set 2 (2A, 2C, and 2D) and a clone each from primer sets 1 (1B), 3 (3C) and 4 (4E) are shown in Figure 2. DcR2 was decreased in the knockdown clones as compared to the control clone.

Figure 2. DcR2 western blot of A2780 control clone (CTR) and knockdown clones (1B, 2A, 2C, 2D, 3C and 4E) with β-Actin loading controls.



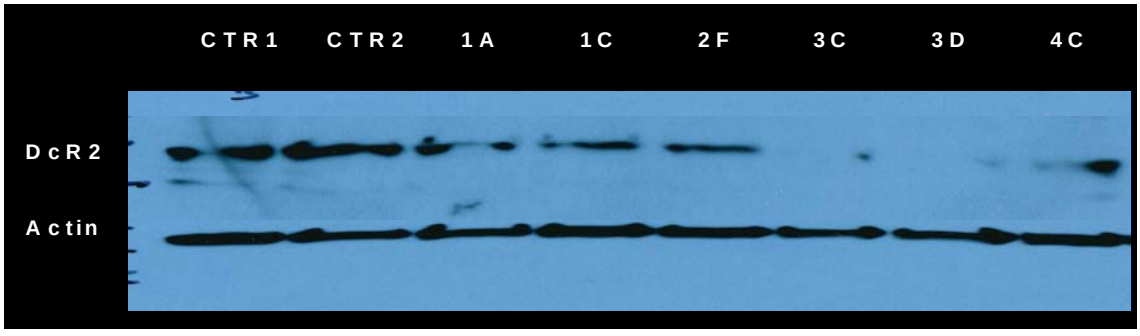
The effect of DcR2 knockdown on sensitivity to TRAIL and agonistic TRAIL antibodies was then studied by performing dose-response assays to TRAIL, FasL and ETR1 and ETR2 using control and DcR2 knockdown clones. Results are shown in Figure 3. DcR2 knockdown shifted the dose-response curve for ETR2 to the left in all cell lines (results show control versus 6 knockdown cell line curves pooled together), although the results were not as dramatic as that seen with Six1 knockdown in the SKOV3 cell lines (Figure 1B). This argues for additional mechanisms for Six1 dependent TRAIL resistance in ovarian cancer. There was no effect on TRAIL, FasL or ETR1 sensitivity (not shown).

Figure 3. DcR2 knockdown sensitizes A2780 cells to ETR2. Dose-response to the agnostic antibody to TRAIL-DR5, ETR2 are plotted as percent of control growth (± Standard Error of the Mean, S.E.M) for the A2780 CTR (control, upper line) cell line and the shRNA DcR2 knockdown clones 1B, 2A, 2C, 2D, 3C and 4E pooled together (Dcr2K/D, lower line). The two curves are significantly different (p=0.02 ANOVA)



In our previously published manuscript, Six1 knockdown sensitized SKOV3 cells to TRAIL. The SureSilencing shRNA knockdown system was also used to generate multiple SKOV3 DcR2 knockdown cell lines using 4 different primer sets (numbered 1-4) and controls. A western blot of DcR2 with controls CTR1 and CTR2, 2 clones from primer sets 1 (1A,1C) and 3 (3C,3D) and a clone from primer sets 2 (2F) and 4 (4C) are shown in Figure 4. DcR2 was decreased in the knockdown clones compared to controls.

Figure 4. DcR2 western blot of SKOV3 control clones (CTR1 and CTR2) and shRNA knockdown clones (1A,1C,2F,3C,3D and 4C) with β-Actin loading controls.



The effect of DcR2 knockdown on sensitivity to TRAIL and agonistic TRAIL antibodies in the SKOV3 DcR2 knockdown clones are currently underway.

Task 3. **Evaluate TRAIL panel sensitivity in Six1 over-expressing and knock-down cells**

A. Generate inducible models of Six1 expression.
B. Perform dose-response curves to TRAIL, FasL, ETR1, ETR2 using existing Six1-CaOV3 over-expression model and Six1 knockdown model, save cell pellets and extract RNA and protein.

In the past year, multiple systems were used to generate both inducible over-expression and inducible knockdown. These included the BD RevTet tetracyclin-on overexpression system and the P30ETREMIRAG lentiviral knockdown system. Neither yielded reproducible and tightly controlled effects as required for this task. While expected to have baseline levels of Six1, controls from the BD RevTet system also over-expressed Six1 suggesting that the system was either "leaky", or the control media contained amounts of tetracycline sufficient to induce the transgene without the addition of any tetracycline. Certified tetracycline free media was ordered and the clones are currently being re-isolated and regrown using this media. The lentiviral knockdown system generated verified expression of the transgene as noted by the presence of a GFP tag on the selected clones, however, Six1 could not be suppressed. A new inducible knockdown system has been successfully used by one of our collaborators and we have secured this system to continue to generate resources for this task

Task 4. **Evaluate TRAIL panel sensitivity in primary ovarian cancer cell lines and correlate with Six1 and *DcR2* expression.**

a. Perform dose-response curves to TRAIL, FasL, ETR1, ETR2 using primary ovarian cancer cell lines, save cell pellets.
b. Extract RNA and protein from cell pellets, correlate with Six1 and DcR2 expression

We have continued to establish cell lines from patients with ovarian cancer and test sensitivity to TRAIL and TRAIL agonistic antibodies. To date, 29 patients have been enrolled and 17 specimens have generated cell lines that could be assayed. Patient specifics and results of the TRAIL, ETR1 and ETR2 are reported in Table 2. All patient specimens were resistant to FasL up to 5000 pg/ml. 6/17 cell lines generated tumors in CB-17 SCID mice and tumorigenicity was associated with Six1 expression ($p=0.02$ χ^2). In year one, we reported no clear developing correlation between Six1 status and TRAIL resistance in primary cell lines derived from patient tumors. This trend continues with the addition of more patients. Since then we have discovered that some primary cell lines rapidly lose Six1 expression in culture and that the cells being tested for TRAIL sensitivity may not be similar to those growing in the patient. Indeed, the tumorigenic cell lines maintain Six1 expression in the mouse tumors while losing Six1 expression in culture. This was anticipated in the original limitations and alternatives section of aim3

and may be the reason behind lack of significance between Six1 expression (reported as tumor Six1) and TRAIL sensitivity in primary tumor specimens (which are tested on the cell lines weeks after culture). As suggested in the alternatives section to aim 3, we plan on studying TRAIL sensitivity in the primary cell lines via establishment of direct (orthotopic) tumors in mice rather than culturing the cells first and then establishing tumors or continuing to study cultured primary cells. This requires more cells from the patient sample initially, but is not a problem with ovarian cancer given the usual large volume of metastatic disease. Table 2 demonstrates the current status of isolated cell lines and results of dose-response curves.

Table 2. Primary cell lines isolated from patients with Age of the patient, Histology of the primary tumor, initial Six1 level, tumorigenicity in CB-17 SCID mice and TRAIL, ETR1 and ETR2 IC_{50}. Resistance was defined as greater than 50 ng/ml for TRAIL and greater than 1000 pg/ml for ETR1 and ETR2. All cell lines were resistant to FasL up to 5000 pg/ml.

#	Age	Stage	Histology	Six1 fg/ng 18s rRNA	Tumors?	TRAIL IC_{50}	ETR1 IC_{50}	ETR2 IC_{50}
159	43	IIIa	Clear Cell	0	No	R	R	R
173	59	IIIb	Serous	7	Yes	R	R	R
140	48	IIIc	Serous	19	No	R	R	R
153	71	IV	Serous	20	No	R	R	R
137	58	IIIc	Serous	58	No	R	R	R
163	52	IIIc	Serous	135	No	2.5 ng/ml	R	200 pg/ml
142	84	IV	Serous	137	No	R	R	R
160	60	IV	Mucinous	205	No	R	R	R
139	56	IIIc	Serous	209	No	R	R	R
150	65	IIIc	Serous	224	Yes	1 ng/ml	R	200 pg/ml
158	45	IIIc	Serous	295	Yes	5 ng/ml	R	600 pg/ml
162	57	IIIc	Serous	301	No	R	R	R
138	52	IIIc	Endo	310	Yes	R	R	R
164	52	IIIc	Serous	324	Yes	1 ng/ml	R	350 pg/ml
167	47	IV	Mixed	410	Yes	R	R	R
161	52	IV	Serous	610	No	5 ng/ml	R	400 pg/ml
141	75	IIIc	Serous	906	No	R	R	R

Task 5. **Establish Syngeneic Six1 over-expression Model**

a. Propagate mouse cell lines and test for Six1 and *DcR2* expression.
b. Establish cell line over-expression and knockdown model and perform dose-response curves to TRAIL, FasL, ETR1 and ETR2.

Ten previously characterized mouse ovarian cancer cell lines (MOSEC) [10] were obtained from Dr. Katherine Roby at the University of Kansas Medical Center under a 3 year Materials Transfer Agreement. MOSEC were tested for Six1 mRNA expression using mouse specific Six1 qRT-PCR primers and probes and results are shown in Table 3. Only the MOSEC IO8 cell line had any significant Six1 expression. DcR2 expression, cell growth, and apoptosis studies to verify the phenotype of Six1 expression in these cell lines are underway.

Table 3. Six1 expression in MOSEC cell lines. Only the IO8 cell line had any significant expression of Six1. Results are reported as fg/ng 18s rRNA.

Cell line	Six1 expression (fg/ng 18s rRNA)
MOSEC 2C6	**3**
MOSEC 2C12	**0**
MOSEC ID9	**0**
MOSEC IO8	**43**
MOSEC ID5	**16**
MOSEC IF5	**5**
MOSEC IC5	**0**
MOSEC 3B11	**0**
MOSEC 3E3	**1**
MOSEC IG10	**0**

Task 6. **Xenograft and/or syngeneic model Six1/*DcR2* over-expression and knockdown analysis.**

a. Test TRAIL, Etoposide (instead of FasL), ETR1 and ETR2 response in xenograft or syngeneic model
b. Evaluate phenotype of in-vivo Six1/*DcR2* knockdown

Our first experiment was to study growth rates of CaOV3 CAT and CaOV3-Six1 transfectants on the flanks of 4-6 week old CB-17 SCID mice. 4 clones total, 4 mice/clone and two tumors/ mouse were initiated by injecting 1×10^7 cells and observing for tumor growth with biweekly measurements of tumor size. Six1 expressing tumors measured 38 ± 6 mm^3 at 2 weeks as compared to 7 ± 3 mm^3 for CAT clones ($p<0.001$ t-test)

demonstrating a faster initial growth rate for Six1 expressing tumors. However tumor growth was poor in the subsequent weeks for both CAT clones and Six1 clones. At 10 weeks Six1 expressing tumors measured 50±19 mm^3 as opposed to 29±11 mm^3 for CAT expressing clones. This difference was no longer significant. A subsequent experiment demonstrated that the CaOV3-Six transfectant xenograft tumors lose Six1 expression within 2 weeks, associated with a decrease in growth rate to baseline. Attempts to study the SKOV3 Six1 knockdown clones in the same system resulted in robust tumor growth in the SKOV3 parental line, but no tumor growth in the SKOV3 Six1 siRNA tumors. These findings, along with the continued growth of patient derived mouse tumors maintaining high Six1 (see task 4) are encouraging because they highlight the importance of Six1 in maintaining tumor growth. However, loss of Six1 overexpression and the lack of tumor growth in knockdown clones makes the study of the effects of treatment in this system difficult. Generation of inducible systems as suggested in task 3 or evaluation in a syngeneic system where exogenous Six1 expression is not lost has the potential to overcome these difficulties.

KEY RESEREARCH ACCOMPLISMENTS:

- Six1 overexpression is associated with TRAIL resistance and over-expression of the TRAIL DcR2 decoy receptor in ovarian cancer cell lines and in a syngeneic over-expression system,
- Six1 knockdown is associated with reversal of TRAIL resistance and decrease in the expression of the TRAIL DcR2 decoy receptor
- Tumorigenicity in ovarian cancer cell lines and in Six1 over-expression and knockdown models appears to be related to Six1 expression.

REPORTABLE OUTCOMES:

The following abstracts have been presented at national meetings as a result of this research:

1. Qamar L, Thorburn A, Davidson SA, **Behbakht K**. Primary ovarian cancers are variably sensitive to TRAIL and Lexatumumab/the agonistic Antibody to TRAIL-Death Receptor 5 but not to Mapatumumab. Presented at the 39th Annual Meeting of the Society of Gynecologic Oncologists, March 2008, published *Gynecol Oncol* 108(2008) page S130.

2. Qamar L, Syed N, Ford HL, Thorburn A and **Behbakht K**. The Six1 homeobox gene is associated with increased Tumor Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL) decoy receptor DcR2 in a Six1 over-expression model. Presented at the 40th Annual Meeting of the Society of Gynecologic Oncologists, February 2009, published *Gynecol Oncol* 112(2009) page S158.

CONCLUSIONS:

Overexpression of the developmental homeobox gene Six1 is gaining importance as a mechanism for carcinogenesis and metastasis is an ever-growing list of malignancies. The list of downstream genes controlled by Six1 is also ever-growing and likely to be tissue specific. We are discovering that overexpression of the TRAIL decoy receptor DcR2 occurs in Six1 overexpressing cancers and that reversing this overexpression can partially sensitize cell to TRAIL agonistic antibodies. Since many current chemotherapies also work by activating TRAIL, this resistance mechanism is likely to have implications on general chemotherapy resistance as well as TRAIL resistance. We plan to conclude our mechanistic and functional studies in the last year of this proposal. These studies will point the way to strategies for reversing the effects of Six1 expression and potentially reversing chemoresistance by blocking downstream targets of Six1 in ovarian cancers.

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10. Roby, K.F., et al., *Development of a syngeneic mouse model for events related to ovarian cancer.* Carcinogenesis, 2000. **21**(4): p. 585-91.

APPENDICES:

Pfd files of presented abstracts are attached.

Results: Twenty-six of 29 patients completed six cycles of chemotherapy. Complete response was attained by 20 of 29 (68.9%) patients. Partial response based on CT evaluation was achieved by four of 29 (13.8%) patients. In one of 29 (3.4%) patients, tumor size increased after the second cycle and then decreased. Two of 29 (6.8%) patients progressed and discontinued treatment: one (1/29, 3.4%) discontinued treatment secondary to carboplatin toxicity, and the other (1/29, 3.4%) patient discontinued treatment secondary to severe thrombocytopenia.
Conclusion: Abraxane can be used to treat recurrent ovarian cancer effectively.

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Port complications associated with delivery of intraperitoneal chemotherapy for ovarian cancer

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Objective: The goal of this study was to examine the complications associated with the use of a single-lumen, cuffless infusaport for delivery of front-line intraperitoneal (IP) chemotherapy to women with ovarian cancer.
Methods: In this retrospective, single-institution study, the records of all women with ovarian cancer (OC) in whom a 9.6F single-lumen Silastic cuffless infusaport (Bard Access Systems) was inserted with intent to deliver intraperitoneal (IP) chemotherapy were reviewed.
Results: The aforementioned infusaport was placed in 38 patients between June 2005 and April 2007. Mean age was 60 (range: 40–81). Of 38 ports, 31 (82%) were placed at the time of initial cytoreductive surgery and seven (18%) were placed postoperatively at a mean interval of 20.6 days (range: 3–54). Twelve of 38 (32%) patients experienced 15 complications associated with the IP port:local pain ($n=4$), port site infection ($n=2$), retraction of catheter tip out of peritoneal cavity ($n=2$), blockage ($n=3$), difficulty accessing the reservoir ($n=3$), colovaginal fistula ($n=1$). There was no association with the use of antiadhesion barriers. Fourteen of 38 patients underwent bowel surgery and two experienced port complications of retraction ($n=1$) and blockage ($n=1$). Sixteen of 38 (42%) patients did not complete intended IP chemotherapy. In six of 16 (37.5%) this was due to port complications: pain ($n=3$), infection ($n=1$) blockage ($n=1$), colovaginal fistula ($n=1$). Three of these patients did not begin IP chemotherapy. Ten of 16 (62.5%) discontinued IP therapy for non-IP port reasons. Five of 38 (13%) underwent an additional surgery to remove and/or change an IP port. Overall, port complications resulted in six of 38 (16%) patients being unable to complete their intended IP chemotherapy.
Conclusions: Complications associated with this IP port are significant. Improved IP delivery techniques are needed.

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The role of fertility-sparing surgery in epithelial ovarian cancer: Oncologic safety and obstetrical outcomes

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Objective: The aim of this study was to evaluate the recurrence, survival, and pregnancy outcome in patients with epithelial ovarian cancer treated with fertility-sparing surgery.
Methods: A retrospective review was performed on patients with epithelial ovarian cancer who underwent fertility-sparing surgery between 1989 and 2007. Fertility-sparing surgery was defined as the preservation of ovarian tissue in one or both adnexa and the uterus. Additional inclusion criteria for this study were (1) age ≤ 40 years, (2) complete staging procedure, and (3) delivery of a platinum-based chemotherapy in high-risk patients. Patients with ovarian tumors of borderline malignancy were excluded.
Results: During the study period, 62 patients fulfilled the inclusion criteria. Thirty-seven patients had stage IA disease, one had stage IB, 21 had stage IC, one had stage IIB, one had stage IIIB, and one had stage IIIC. Histologic types of tumor were as follows: mucinous, 41; serous, seven; endometiroid, eight; clear, four; mixed, two. Histologic grades of tumor were as follows: grade 1, 48; grade 2, five; grade 3, nine. Forty-eight patients received platinum-based adjuvant chemotherapy (mean=4.6 cycles, range: 1–9 cycles). Five patients had second-look laparotomy and all were negative. The median follow-up time was 59 months (range: 6–205 months). Eleven patients had a recurrence six-58 months after initial surgery. Among 11 patients with recurrence, initial stage and grade were: stage IA grade 1, $n=1$; stage IA grade 3, $n=4$; stage IC grade 1, $n=1$; stage IC grade 2, $n=1$; stage IC grade 3, $n=2$; stage IIIB grade 1, $n=1$; stage IIIC grade 3, $n=1$. Histologic types of tumor were: mucinous, seven; clear cell, two; endometrioid, one; and mixed, one. Five patients underwent secondary cytoreductive surgery including hysterectomy and salpingo-oophorectomy followed by adjuvant chemotherapy. Six patients received second-line chemotherapy. At the end of the study, six patients died of disease, two patients were alive with disease, and 54 patients were alive without disease. One patient suffered from infertility because of low ovarian reserve after primary treatment. Eight patients achieved 15 pregnancies resulting in 13 term deliveries and two spontaneous abortions.
Conclusions: Fertility-sparing surgery for patients with epithelial ovarian cancer could be considered in young patients with stage IA grade 1 or 2 who desire fertility preservation. However, this procedure is not recommended in patients with more advanced stage, higher grade, or unfavorable histologic type of tumor.

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Primary ovarian cancers are variably sensitive to TRAIL and lexatumumab/the agonistic antibody to TRAIL death receptor 5, but not to Maptumumab

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Objective: TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) and agonistic antibodies to TRAIL death receptors 4 and 5 (DR4 and DR5) are currently in phase I trials. These trials are supported by preclinical data indicating these compounds exhibit toxicity in tumor cells while sparing normal

cells, yet the degree of sensitivity of primary ovarian cancers or the optimum agent is not known. The objective was to evaluate the sensitivity of normal and malignant ovarian cancer cell lines and primary cell lines from patient tumors to TRAIL, FasL, and the TRAIL agonistic antibodies Maptumumab (agonistic antibody to TRAIL receptor 1_DR4) and Lexatumumab (agonistic antibody to TRAIL receptor 2_DR5).

Methods: Ovarian cancer cell lines, an immortalized normal ovarian surface epithelial cell line, and 14 cell lines generated from 18 patients undergoing surgery for stage III/IV ovarian cancer were included. Primary cell lines survived four to six passages before senescence, and none were immortal. Dose-response curves were generated to native sequence TRAIL (to 1000 ng/mL), FasL (to 5000 pg/mL), Maptumumab (to 1000 ng/mL), and Lexatumumab (to 1000 ng/mL). Dose responses (IC_{50}) were compared with respect to extent and location of disease at surgery and extent of residual disease.

Results: Mean age of patients whose tumors generated successful cell lines was 58 years (range: 39–84). Tumors were 80% stage III (11/14) and 80% serous histology (11/14). Twenty-eight percent (4/14) were sensitive to TRAIL (IC_{50} <5 ng/mL), and none were sensitive to FasL. All primary cell lines sensitive to TRAIL were also sensitive to Lexatumumab (IC_{50} <200 ng/mL) but not to Maptumumab. Patients whose cell lines were sensitive to TRAIL/Lexatumumab had smaller residual disease after surgery ($P=0.04$, Mann-Whitney U test). All TRAIL-resistant cells were also resistant to Maptumumab. The majority of established ovarian cancer cell lines were resistant to TRAIL, FasL, and the antibodies.

Conclusions: Primary cell lines generated from ovarian cancers are variably sensitive to TRAIL, and TRAIL sensitivity correlates with surgical success in this study sample. Sensitivity to TRAIL correlates with Lexatumumab but not Maptumumab sensitivity. These findings have important implications for further studies selecting the proper patients and the proper TRAIL antibody for clinical trials. Analysis of TRAIL DR4/DR5 expression in ovarian tumors and its relationship to therapy response is underway.

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The clinical significance of p130cas in ovarian carcinoma

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Objective: p130cas is a key converging point for signals from many growth factor receptors and integrins and has been implicated in malignant transformation and cancer progression. The purpose of this study was to address the role of p130cas in ovarian carcinoma.

Methods: Western blot analyses were used to determine p130cas expression in ovarian cancer cell lines. After institutional review board approval, p130cas expression in 91 invasive epithelial ovarian cancers was examined by immunohistochemistry. Clinical data were extracted from the M.D. Anderson Cancer Center Tumor Bank.

Results: p130cas was minimally expressed in the nontransformed HIO-180 ovarian epithelial cells, but was detected at high levels in the HeyA8, HeyA8-MDR, SKOV3ip1, OVCAR, IGROV, and 222 ovarian cancer cell lines. The mean age of patients was 61 (range: 36–89). Eighty percent of patients had serous histology, and 87% patients had high-grade histology. Eighty-three percent of patients had advanced-stage disease, and 74% had associated ascites. p130cas overexpression was associated with advanced-stage (III or IV) disease ($P<0.001$) and suboptimal cytoreduction ($P=0.007$). Multivariate analysis was performed using a Cox proportional hazard model and revealed that advanced age ($P=0.02$), advanced stage ($P=0.02$), suboptimal cytoreduction ($P=0.002$), and p130cas overexpression ($P=0.001$) were independent predictors of poor survival. Patients with p130cas overexpression had a median survival of 2.14 months, compared with 9.1 months for those with low expression ($P<0.001$).

Conclusions: p130cas overexpression is associated with several aggressive tumor features in patients with ovarian carcinoma. Based on the findings and its prominent role in tumor cell function, p130cas may represent an attractive therapeutic target.

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Expression of peroxisome proliferator-activated receptors α, β, and γ in ovarian carcinoma effusions is associated with response to chemotherapy and poor survival

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Objectives: The aims of this study were to analyze the expression and prognostic role of peroxisome proliferator-activated receptors (PPARs) α, β, and γ in ovarian carcinoma effusions and to investigate the anatomic site-related expression of PPAR-γ and COX-2.

Methods: Fresh-frozen malignant effusions ($n=79$) from patients diagnosed with ovarian carcinoma were studied for mRNA expression of the three PPARs using reverse transcription polymerase chain reaction. Results were analyzed for possible correlations with clinicopathologic parameters. The anatomic site-related expression of PPAR-γ and COX-2 mRNA was studied in 106 specimens (35 effusions, 71 solid tumors) using mRNA in situ hybridization (ISH).

Results: PPAR-α, -β, and -γ were expressed in 79 of 79 (100%), 70 of 79 (89%), and 75 of 79 (95%) effusions, respectively, and their expression levels were strongly interrelated ($P<0.001$). PPAR expression was higher in effusions from patients who responded poorly to chemotherapy at disease recurrence ($P=0.009$ for all three molecules). In univariate survival analysis, higher expression of all PPAR members was associated with poor progression-free ($P=0.045$) and overall ($P=0.014$) survival. The clinical parameters associated with poor survival were response to chemotherapy at diagnosis ($P<0.001$ for overall and disease-free survival) and at disease recurrence

Conclusions: Clinicians struggle in their efforts to distinguish patients with recurrent ovarian cancer who have potentially reversible and treatable problems from those who are entering a terminal phase of their illness. In the final 100 days of an ovarian cancer patient's life, the disease produces distinct symptoms requiring management and resource utilization. Our data suggest that even as disease progresses, we are inclined to perform evaluations and offer treatments, as well as offer care to provide symptom management. Worsening gastrointestinal symptoms or increased use of hospital admission or procedures should identify patients as potentially moving toward the final phases of their illness.

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The search for meaning, symptoms and transvaginal ultrasonography screening for ovarian cancer: Predicting malignancy

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Objectives: The mortality rate of ovarian cancer is greater than that of all other major gynecologic malignancies. Most women present with advanced-stage disease, where response to treatment is limited and prognosis is poor. Detecting ovarian cancer at an early stage, when it is curable, has long been an important goal of gynecologic oncologists. Recently, it has been reported that certain symptom patterns can be informative for the presence of ovarian malignancy. The present investigation was performed to determine how well symptoms and ultrasound findings would predict ovarian malignancy individually or in combination.

Methods: A group of 450 women, all of whom received surgery due to participation in annual transvaginal ultrasonography (TVS) screening, were selected from 31,748 women enrolled. Symptom questionnaires were provided, and the tabulated results were compared with ultrasound reports and surgical pathology for 272 of the women.

Results: Thirty malignancies and 420 persisting benign tumors constituted the group under study. The ability to distinguish malignant from benign ovarian tumors was based on sensitivities, specificities, and ROC curve analysis. TVS performed better than symptom analysis for detecting malignancies (73.3% vs 20% sensitivity), and symptom analysis performed better for distinguishing benign tumors (91.3% vs 74.4% specificity). Decisions based on simultaneously meeting TVS and symptom criteria resulted in poorer identification of malignancy in ROC analysis (with Morphology Index (MI) >5 and symptom analysis, sensitivity=16.7%), but improved the ability to distinguish benign tumors (with MI>5 and symptom analysis, specificity=97.9%). Decisions based on satisfying either symptom criteria or TVS criteria had small increases in sensitivity (+3.3%) and coordinated small decreases in specificity (-5.8%).

Conclusions: Symptom analysis does identify malignant ovarian tumors, but its discrimination by itself is inferior to that of TVS. The clinical significance of the findings reported here is that: (1) a screen that is negative by both ultrasound and the symptom index is likely to indicate a benign tumor (specificity >97%), and (2) adding symptom information with equal weight as ultrasound slightly improves the discrimination of malignancy (one additional TP with a sensitivity increase=+3.3%). These results strongly indicate that the major screening benefit in discriminating malignancy is achieved via ultrasound tools, whereas symptom information can aid in reducing surgery on women with benign conditions that generate ultrasound abnormality. Combining symptom analysis with TVS improved the discrimination of benign tumors, but it is coordinated with much poorer discrimination of malignant tumors, indicating that informative symptoms can be expected to be absent in a large fraction (80%) of ovarian malignancies.

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The six1 homeobox gene is associated with resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in ovarian cancers and is correlated with increased TRAIL decoy receptor DcR2 in a six1 overexpression model

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Objectives: Ovarian cancers express TRAIL receptors and TRAIL synergizes with chemotherapy in ovarian cancers. However, up to 60% of ovarian cancers overexpress the Six1 homeobox gene and we have shown that Six1-overexpressing ovarian cancers are resistant to TRAIL. To assess the role of TRAIL decoy receptors in Six1-related TRAIL resistance, we studied the expression of TRAIL and TRAIL decoy receptors and correlated these with Six1 expression and dose response to TRAIL and TRAIL receptor agonists.

Methods: Six1 expression and TRAIL receptor DR4 and DR5 and decoy receptor DcR1 and DcR2 mRNA levels were analyzed by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) in a panel of 15 ovarian cancer cell lines as well as Six1 stable transfected CaOV3 clones and control CAT clones. Dose-response curves were generated to TRAIL, FasL (as a control for non TRAIL receptor-induced apoptosis)and agonistic antibodies to TRAIL DR4 and DR5 and correlated with Six1 and TRAIL receptor expression.

Results: All 15 cell lines expressed DR4 (mean=123±77 ag/ng rRNA), DR5 (mean=210±106 ag/ng rRNA) and DcR2 (mean=81±63 ag/ng rRNA), but only once cell line expressed DcR1. Six1 expression (overexpression vs underexpression, mean=108 fg/ng rRNA, range: 0-763) correlated with TRAIL resistance (TRAIL IC_{50}>100 ng/mL, $P=0.05$, χ^2 test) and all cell lines sensitive to TRAIL were also sensitive to anti-DR5

antibody (IC_{50}<1000 ng/mL), but not sensitive to anti-DR4 antibody. Cells were resistant to FasL. Although Six1 mRNA compared across all cell lines did not correlate with DcR2 expression, stable Six1 overexpression in the low-Six1, low-DcR2-expressing CaOV3 cell line increased TRAIL IC_{50} fivefold and significantly increased DcR2, whereas DcR1 levels were unchanged.

Conclusions: Ovarian cancer cells express TRAIL DR4 and DR5 and the decoy receptor DcR2. Decoy receptor DcR1 expression is uncommon. Six1 expression correlates with DcR2 expression and TRAIL resistance in a CaOV3 Six1 over-expression model. The Six1-correlated increase in the TRAIL decoy receptor DcR2 may be a mechanism for TRAIL resistance in ovarian cancers. Given the relationship between Six1 expression and TRAIL resistance, the lack of a direct correlation between Six1 and DcR2 across all cell lines implies other Six1-driven TRAIL resistance mechanisms as well. Additional Six1 overexpression and knockdown experiments are underway.

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The utility of physical examination in detecting recurrence in patients with advanced epithelial ovarian cancer

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Objectives: The purpose of this study was to assess the value of physical examination in detecting recurrence in patients with advanced epithelial ovarian cancer in complete remission.

Methods: This was a retrospective study of patients with stage IIIC and IV epithelial ovarian cancer diagnosed between 1997 and 2005 who underwent primary surgical debulking followed by adjuvant chemotherapy. We included only patients who had a complete response to adjuvant chemotherapy with no evidence of disease on physical examination, CA-125 determination, and CT scan (when available) and who suffered from recurrence of their disease while under our care.

Results: Seventy-nine patients fit the inclusion criteria. Median age was 59.8 (range: 30-89). Seventy-one patients (89.9%) had stage IIIC and eight (10.1%) had stage IV. Seventy-four patients (93.7%) had papillary serous, two (2.6%) had clear cell, two (2.6%) had endometrioid, and one (1.3%) had mucinous adenocarcinoma. Seventy-seven patients (97.5%) had grade 3, one patient had grade 2, and one had grade 1. Preoperative CA-125 levels were available for 74 patients with a median of 537 (range: 17-25,224) U/mL; six of the 74 had normal preoperative levels (<35 U/mL). The first evidence of recurrence was CA-125 elevation in 62 patients (78.5%), positive clinical findings on physical examination in 9 patients (11.4%), positive CT scan in seven patients (8.9%), and one patient was incidentally found to have recurrent carcinoma in the hernia sac during hernia repair. Of the 9 patients who were first diagnosed with recurrence based on positive clinical findings, seven (77.7%) had significant symptoms that prompted the physical examination (two had bowel obstruction, two had neurologic symptoms, one had flank pain, one had a groin mass, one had a new large breast mass). Two patients had asymptomatic recurrences first found on physical examination during a routine follow-up visit; however, one had an elevated CA-125 and the other had an abnormal CT scan and both of these tests were already scheduled on the same day as the physical exam.

Conclusions: Physical examination has limited utility in detecting ovarian cancer recurrence during routine follow-up visits. Patients with an initial clinically diagnosed recurrence either were symptomatic or had concurrent positive routine CT scan or CA-125. Changing the routinely scheduled follow-up visits to an as-needed basis may be more convenient and economical in patients with epithelial ovarian cancer in remission.

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Therapeutic efficacy of folate receptor α blockade with MORAb-003 in ovarian cancer

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Objectives: The relative overexpression of folate receptor α (FRα) in ovarian cancer compared with normal tissues offers opportunities for novel therapeutic approaches to ovarian cancer. The purpose of this study was to examine the functional significance of FRα blockade with a novel monoclonal antibody, MORAb-003.

Methods: FRα expression was examined in ovarian cell lines (SKOV3ip1, IGROV, HeyA8, A2780-par, and HIO-180) with fluorescence-activated cell sorting analysis. In vitro (cell viability, migration, invasion) and in vivo (tumor growth) effects of FRα blockade on ovarian cancer cells were examined using well-characterized models. The mechanistic effects on the src-family nonreceptor tyrosine kinase Lyn were also examined.

Results: IGROV and SKOV3ip1 cell lines both expressed high levels of FRα compared with the non-transformed (HIO-180) cells. HeyA8 and A2780-par cell lines lacked FRα expression. In vivo, MORAb-003 led to 44 and 84% decreases in tumor growth in SKOV3ip1 and IGROV, respectively, when compared with control IgG antibody. Compared with other groups, the greatest efficacy was noted in the MORAb-003 plus docetaxel group (96 and 99% decreased tumor growth for SKOV3ip1 and IGROV compared with controls, $P<0.001$). In the IGROV model, treatment with MORAb-003 resulted in a 27% decrease in tumor cell proliferation by PCNA staining ($P<0.001$). MORAb-003 redistributed active, phosphorylated Lyn kinase out of lipid rafts, with a 60% decrease in active Lyn compared with control antibody. MORAb-003 did not significantly affect SKOV3ip1 cell viability, migration or invasion in vitro.